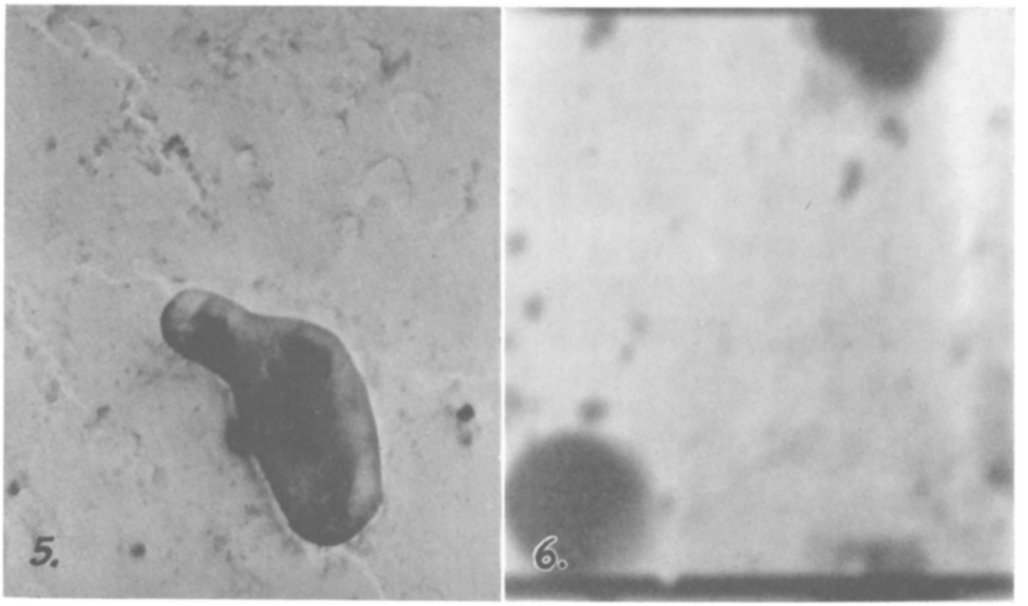

FIG. 5. Swollen cell of *M. tuberculosis* strain 607 after 15 min. contact with phage concentrate. × 36514.

FIG. 6. Protoplasts of *M. tuberculosis* strain 607 after 60 min. contact with phage concentrate. × 25025.

sufficiently high phage bacteria ratio. This was accomplished by lyophilization. The significance of the reported findings is discussed.

1. Youmans, G. P., Millman, I., Youmans, A. S., *J. Bact.*, 1955, v70, 557.

2. Millman, I., Darter, R. W., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 271.

3. Darter, R. W., Millman, I., *ibid.*, 1957, v95, 440.

4. Youmans, A. S., Youmans, G. P., Millman, I., *ibid.*, 1957, v96, 762.

5. Millman, I., Youmans, G. P., *J. Bact.*, 1955, v69, 320.

6. Cota-Robles, E. H., Marr, A. G., Nilson, E. H., *ibid.*, 1958, v75, 243.

7. Sohler, A., Romano, A. H., Nickerson, W. J., *ibid.*, 1958, v75, 283.

8. Fitz-James, *ibid.*, 1958, v75, 369.

9. Carey, W. F., Spilman, W., Baron, L. S., *ibid.*, 1957, v74, 543.

Received July 10, 1958. P.S.E.B.M., 1958, v99.

o-Hydroxyphenylacetic Acid Excretion in the Phenylalanine Tolerance Test For Carriers of Phenylketonuria.* (24301)

ANDREA M. CULLEN AND W. EUGENE KNOX (Introduced by Shields Warren)

Cancer Research Institute, New England Deaconess Hospital and Dept. of Biology and Chemistry, Harvard Medical School, Boston, Mass.

The recessive gene responsible for phenylketonuria is associated in the heterozygous parents with an abnormally low tolerance for ingested phenylalanine(1) and an elevated fasting level of plasma phenylalanine(2). This accumulation of phenylalanine in the apparently normal carriers of the disease is referable to a partial impairment of phenylalanine hydroxylase, the enzyme whose ab-

* Support is acknowledged from U. S. Atomic Energy Comm., to New England Deaconess Hospital and U. S. Public Health Service.

sence from the liver of the homozygous phenylketonuric individuals(3,4,5) may account for all of the biochemical and clinical abnormalities of the disease(6). The reduced phenylalanine tolerance of carriers of the phenylketonuric gene was confirmed by Berry, Sutherland and Guest(7), who also reported that o-hydroxyphenylacetic acid (o-HPA) was excreted in the urine of the heterozygotes, but not of normals, after phenylalanine was administered in test doses. No other metabolites characteristic of phenylketonuria were found regularly in these experiments. The suggestion was made that the presence of o-HPA in urine during tolerance tests could perhaps serve as an added means of identifying the carriers of the recessive gene. o-HPA is the major abnormal hydroxyphenyl metabolite excreted in phenylketonuria(8) and its increased formation has been attributed to the action of the same genetically controlled enzyme whose normal *para*-hydroxylation of phenylalanine is impaired(9). If this were so, its appearance in the urine of heterozygotes would represent a qualitative test for the presence of the genetically distorted enzyme reaction, a test superior to the quantitative tests now used. It would also indicate the kind of chemical alteration which affects the enzyme in this disease. On the other hand, o-HPA formation may occur by an independent system in proportion to the height of the blood phenylalanine level. In phenylketonuria the excretion of o-HPA is proportional to the blood phenylalanine level(8). The following experiments demonstrate that the same proportionality of o-HPA excretion to blood phenylalanine level holds in normal individuals, and that o-HPA formation is not qualitatively dependent upon the presence of the phenylketonuric gene.

Methods. Four young adult members of the laboratory staff, 3 males and 1 female, were subjects. All were non-heterozygotes as judged by normal basal levels of plasma phenylalanine, and 2 of these, previously tested, showed normal phenylalanine tolerance curves. The curve on one of these (PI) was at the high extreme of the normal range. In the present study these subjects were given by mouth an amount of phenylalanine calculated to raise the phenylalanine in their blood to a level comparable to that which the heterozygote would reach with 0.1 g/kg. The subjects were not fasting. After ingestion of the designated amount of L-phenylalanine, blood samples and urine specimens were obtained at hourly intervals for 3 hours. Phenylalanine in plasma was determined by a modification(2) of the decarboxylase method of Udenfriend & Cooper(10). o-HPA was measured by paper chromatography of ether extracts of the urine, following the method described by Berry, Sutherland and Guest (7). The only modification of this technic consisted in using twice the amount of concentrated urine extract (equivalent to 400 μg of creatinine) for each spot on the chromatograms.

Results. We have confirmed the findings of Berry, Sutherland and Guest(7) on 14 heterozygotes and 9 controls that after 0.1 g/kg of L-phenylalanine usually only the heterozygotes excrete detectable amounts of o-HPA. The blood levels of phenylalanine expected after this dose of phenylalanine in the two types of individuals are shown by the dotted lines in Fig. 1, representing the

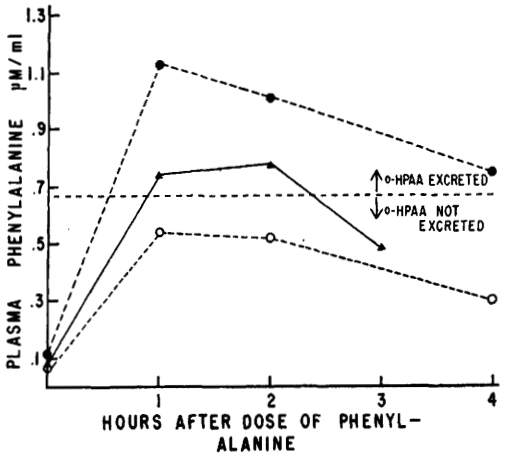

FIG. 1. Plasma phenylalanine following oral doses of L-phenylalanine in 19 controls (–○–) and 19 heterozygotes (–●–) given 0.1 g L-phenylalanine per kg of body wt, and in 4 normal subjects (–▲–) given 0.1 to 0.13 g L-phenylalanine per kg body wt (Table I). Dotted line at 0.67 μm/ml represents "threshold" of plasma phenylalanine above which excretion of o-HPA was observed by the method used.

TABLE I. Plasma Phenylalanine and Urine o-HPA.

Subject	Phenylalanine ingested, g/kg body wt	Plasma phenylalanine, μM/ml (after ingestion of phenylalanine)				o-HPA excreted in 3 hr, μg/mg creatinine
		Fasting	1 hr	2 hr	3 hr	
TA	.13	.072	.804	.858	.549	18
PI	.1	.068	.935	.776	.426	21
BE	.13	.075	.544	.803	.489	3
CU	.1	.080	.679	.495	.400	trace
"	.12		.691	.672	.459	4

average curves for 19 individuals in each group(1). Superimposed on this graph is the average of the tolerance curves of the 4 normal subjects after receiving the doses of L-phenylalanine listed in Table I. During the 3 hour period following ingestion all subjects excreted 3 to 21 μg of o-HPA per mg of creatinine. The horizontal line at about 0.67 μmoles/ml represents the "threshold" level of plasma phenylalanine. With values above this level the excretion of o-HPA measured by the present method regularly occurred in both normal and heterozygote individuals. The amount of o-HPA excreted was roughly proportional to the area of the curve above this line. A similar division between the plasma levels of those who did and those who did not excrete o-HPA is seen at about 9 mg% in the data of Berry, *et al.* This and the other values determined by their method were about 30% less than by our method. The excretion of about 1 mg o-HPA per day by normal individuals(8) indicates that more sensitive methods than we employed would detect o-HPA in the urine of individuals whose phenylalanine levels were below the "threshold" line.

In Table I the individual phenylalanine levels of the 4 subjects are given with the sum of o-HPA excreted by each during the 3 hour test period. It is to be noted that normal subject, PI, whose tolerance curve was at the upper range of normal despite a normal basal level, excreted o-HPA after the standard dose of phenylalanine of 0.1 g/kg body weight. Subject CU excreted significant amounts of o-HPA only with a dose of 0.12 g/kg and not after 0.1 g/kg. Even in the latter case traces of o-HPA were found which would not have been detectable with the smaller aliquots chromatographed by Berry, Sutherland and Guest(7).

Discussion. While the heterozygote and the homozygous phenylketonuric have a deficiency in an enzyme system responsible for the metabolism of phenylalanine, they are not unique in disposing of an excess of phenylalanine in part by o-hydroxylation. That this also occurs in the normal is shown in the present work. Under conditions which impose a comparable load of phenylalanine in the blood of the non-heterozygote, the latter also has the capacity to form o-HPA. Isotope studies in the phenylketonuric patient showed that o-HPA was derived from phenylalanine(11). The results of the present study indicate that o-HPA is also derived from phenylalanine in normals and carriers of the phenylketonuric gene. This must occur by another reaction(12), and not by distortion of the phenylalanine hydroxylase reaction. In the presence of elevated blood levels of phenylalanine this second reaction functions as one of the overflow mechanisms to dispose of the excess amino acid. The excretion of o-HPA during a phenylalanine tolerance test reflects an elevated blood level of phenylalanine, and while it is an additional indication of a low tolerance, it is not independent evidence of heterozygosity for phenylketonuria.

Summary. o-Hydroxyphenylacetic acid is an abnormal urinary metabolite of phenylalanine found in phenylketonuria and after phenylalanine dosage in heterozygotes of phenylketonuria. It is also formed by normal individuals with comparably elevated phenylalanine blood levels. It must be formed by enzyme systems other than the one disturbed in phenylketonuria.

1. Hsia, D. Y., Driscoll, K. W., Troll, W., Knox, W. E., *Nature*, 1956, v178, 1239.

2. Knox, W. E., Messinger, E. C., *Am. J. Hum. Gen.,* 1958, v10, 53.

3. Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 514.

4. Mitoma, C., Auld, R. M., Udenfriend, S., *ibid.,* 1957, v94, 634.

5. Wallace, H. W., Moldave, K., Meister, A., *ibid.,* 1957, v94, 632.

6. Knox, W. E., Hsia, D. Y., *Am. J. Med.,* 1957, v22, 687.

• 7. Berry, H., Sutherland, B., Guest, G. M., *Am. J. Hum. Gen.,* 1957, v9, 310.

8. Armstrong, M. D., Shaw, K. N. F., Robinson, K. S., *J. Biol. Chem.,* 1955, v213, 797.

9. Armstrong, M. D., Shaw, K. N. F., *ibid.,* 1955, v213, 805.

10. Udenfriend, S., Cooper, J. R., *ibid.,* 1953, v203, 953.

11. Udenfriend, S., Mitoma, C., in *Symposium on Amino Acid Metabolism,* ed. by McElroy, W. D., Glass, B., Baltimore, 1955, p876.

12. ———, *et al.,* in *Symposium on Enzymatic Activation of Molecular Oxygen,* Abst. Am. Chem. Soc., 1956, Sept. 16-21, p53c.

Received July 11, 1958. P.S.E.B.M., 1958, v99.

Studies in Leukemia: XI. Active Immunization of C3H x 101 Mice Against Induction of Leukemia.* (24302)

BILL P. MADUROS, STEVEN O. SCHWARTZ AND HAROLD M. SCHOOLMAN

Dept. of Hematology, Hektoen Institute for Medical Research, Cook Co. Hospital, Chicago, Ill.

Our previous reports have described induction of leukemia in C3H mice by means of cell-free brain filtrates(1). Experiments were devised to study the possible immunologic properties of cell-free brain filtrates exposed to ultraviolet light. The present report gives the results of experiments regarding active immunization of mice against leukemia.

Materials and methods. C3H x 101 mice, from 8 to 10 weeks old, served in the experiments. The mice were obtained from Cumberland View Farms, Clinton, Tenn. Cell-free brain filtrates (CFBF) were prepared by method previously described(2). Tumor-cell suspensions were prepared by grinding mesenteric tumor in ground glass tissue mill and suspending the cells in a 0.9% solution of sodium chloride, buffered at pH 7.4, to a concentration of about 10^6 cells/cm^3. Brains of 5 leukemic C3H x 101 mice were pooled; 1000 cc. of CFBF was prepared. A control filtrate was similarly prepared with brains of non-leukemic C3H x 101 mice. The filtrates were stored for 48 hours at −15°C, then thawed 24 hours at 4°C. After filtrates had thawed, the non-leukemic CFBF was filtered through a coarse and a fine glass-sintered filter; the leukemic CFBF was filtered through the same filters. Samples of leukemic CFBF taken before and after this filtration retained their leukemogenic properties when later tested. The filtrates were irradiated with ultraviolet light (UVI) in a Spinco centrifilmer irradiation machine at 1750 rpm at full power and rate of flow of 200 ml/minute.† When irradiation was accomplished, the filtrates were stored in 5 ml and 10 ml vials at −60°C until used. All experimental mice were immunized with ultraviolet irradiated cell-free brain filtrate (UVI-CFBF) by subcutaneous inoculations of 0.1, 0.25, and 0.5 cc at 48-hour intervals. The mice were challenged 10 days after last inoculation, by intraperitoneal (IP) injection of 0.5 cc CFBF or tumor-cell suspension prepared from leukemic C3H x 101 mice. The mice were killed when they appeared terminally ill and autopsies performed. In mice described as positive, both gross and microscopic data regarding leukemia were similar to those described elsewhere(3) for the Swiss strain. Ten C3H x 101 mice were

* These studies were aided by grants from Am. Cancer and U. S. Public Health Service.

† Irradiation of filtrates was supervised by Dr. A. M. Wolf and his staff at Michael Reese Research Fn.